SNARE Proteins in Synaptic Vesicle Fusion

Mark T. Palfreyman, Sam E. West, and Erik M. Jorgensen

Abstract Neurotransmitters are stored in small membrane-bound vesicles at synapses; a subset of synaptic vesicles is docked at release sites. Fusion of docked vesicles with the plasma membrane releases neurotransmitters. Membrane fusion at synapses, as well as all trafficking steps of the secretory pathway, is mediated by SNARE proteins. The SNAREs are the minimal fusion machinery. They zipper from N-termini to membrane-anchored C-termini to form a 4-helix bundle that forces the apposed membranes to fuse. At synapses, the SNAREs comprise a single helix from syntaxin and synaptobrevin; SNAP-25 contributes the other two helices to complete the bundle. Unc13 mediates synaptic vesicle docking and converts syntaxin into the permissive "open" configuration. The SM protein, Unc18, is required to initiate and proofread SNARE assembly. The SNAREs are then held in a half-zippered state by synaptotagmin and complexin. Calcium removes the synaptotagmin and complexin block, and the SNAREs drive vesicle fusion. After fusion, NSF and alpha-SNAP unwind the SNAREs and thereby recharge the system for further rounds of fusion. In this chapter, we will describe the discovery of the SNAREs, their relevant structural features, models for their function, and the central role of Unc18. In addition, we will touch upon the regulation of SNARE complex formation by Unc13, complexin, and synaptotagmin.

Keywords SNARE · Munc18 · Munc13 · Unc18 · Unc13 · Complexin · Synaptotagmin · SNAP-25 · Synaptobrevin · Syntaxin · NSF · Membrane fusion · Synaptic vesicle

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Abbreviations

CATCHR	Complexes Associated with Tethering Containing Helical Rods
NSF	NEM-sensitive factor
RIM	Rab3-interacting molecule
SM proteins	Sec1/Munc18
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
Unc13	Uncoordinated-13
Unc18	Uncoordinated-18

1 SNARE DISCOVERY—A Convergence of Genetics and Biochemistry

To understand the mechanisms of synaptic vesicle fusion, it is useful to think about the evolution of neurotransmission. In prokaryotic cells, the cytoplasm comprises a single compartment, which limits the diversity of potential chemical reactions conducted in a cell. By contrast, eukaryotic cells segregate cellular functions into specialized membrane-bound compartments, or organelles. The contents of these organelles are moved between compartments by transport vesicles. To transfer cargo, the lipid bilayers of the vesicle and target organelle must fuse. Fusing negatively charged membranes is an energetically unfavorable process. SNARE proteins evolved to force membranes together and merge them, so that the cargo in the transport vesicle is transferred to the lumen of the target organelle. In some cases, cargo must be secreted into the extracellular space via exocytosis, in particular to signal to other cells in the environment. During evolution, it was perhaps a small step to couple SNARE-mediated fusion to membrane depolarization, but it was a giant leap for the diversity of life—the nervous system is arguably the universe's greatest invention.

The identification of SNAREs as the central players in membrane fusion arose from a convergence of independent scientific approaches: protein purification from brain, genetic studies in yeast, pharmacological approaches from toxicology, electrophysiological approaches in model organisms, and in vitro reconstitution assays for membrane fusion.

In the late 1980s, SNARE proteins were identified in the brain as components of the synapse. Specifically, synaptobrevin (also called VAMP—vesicle-associated membrane protein) was purified from synaptic vesicles from the electric ray *Torpedo* [1]. The other two SNARE proteins, syntaxin and SNAP25 (synaptosomal-associated protein of 25 kDa), were purified from rat brain [2–5]. The identification of homologs among the yeast *sec* genes (*sec*retion defective) linked the mechanisms of synaptic function to vesicular trafficking [6, 7] and hinted at the universality of membrane fusion in the trafficking pathways of all eukaryotic cells. However, at

this point there was no evidence that these proteins functioned in calcium-dependent exocytosis of synaptic vesicles. Evidence these proteins were required for neurotransmission came from the study of toxins found in bacteria.

Clostridia are anaerobic soil bacteria that can cause fatal infections in animals. A bizarre feature of such infections is that they produce toxins that can cause muscle paralysis lasting many days [8]. The groups of Heiner Niemann, Reinhard Jahn, and Cesare Montecucco identified the targets of the clostridial toxins at synapses. It was found that botulinum and tetanus toxins cleave synaptobrevin, syntaxin and SNAP25 demonstrating the central role of these proteins in synaptic vesicle release [9–12]. These were the first functional data that the SNAREs were involved in neurotransmission [13, 14]. The essential role of the SNAREs in neurotransmission would later be demonstrated from electrophysiological studies on null mutants in the SNARE proteins in *Drosophila*, mice, and *C. elegans* [15–20]. Thus, the functional data demonstrated that each of these SNARE proteins are required components of synaptic transmission, but their physical association as a complex was not yet known.

The discovery that these proteins formed a complex was demonstrated by experiments aiming to reconstitute membrane fusion. Jim Rothman's group was taking a biochemical approach to understand trafficking in the Golgi apparatus. The toxin N-ethylmaleimide (NEM) potently blocks Golgi trafficking [21, 22] by inhibiting NSF (NEM sensitive factor) [23], the mammalian homolog of the yeast gene *SEC18* [24–26]. NSF was found to bind, via the action of the soluble NSF adaptors (SNAPs) [27], to a set of proteins from brain detergent extracts called SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptor) [28]. When these protein complexes were analyzed by mass spectroscopy, it was found that they comprised synaptobrevin, syntaxin, and SNAP25 (Fig. 1a). The perturbation experiments described above, combined with their physical association, convincingly linked these proteins to synaptic vesicle exocytosis, but a list of names in a complex did not constitute a model.

The first coherent model, called the "SNARE hypothesis," would arise from the melding of the genetic and biochemical observations described above. Although wrong in detail, it would catalyze a number of hypothesis-driven experiments that would lead to more accurate models. Based on the finding that a unique set of SNARE proteins were found at each of the trafficking steps [29, 30], Thomas Söllner and Jim Rothman proposed that SNARE interactions provided the specificity for vesicular trafficking by tethering the vesicle to its target membrane—essentially providing an addressing system within the cell [28, 31]. The SNAREs would then be acted on by the ATPase NSF which, by disassembling the SNAREs, would drive fusion [31, 32].

Experiments from Bill Wickner's lab, using a purified vacuole fusion assay, demonstrated that NSF acts not at the final step of fusion, but rather to recover monomeric SNAREs for use in further rounds of fusion [33–35]. Studies at synapses indicated that NSF acts after membrane fusion during the recovery of synaptic vesicles [36, 37]. These data indicated that SNARE assembly, not disassembly, catalyzes fusion.

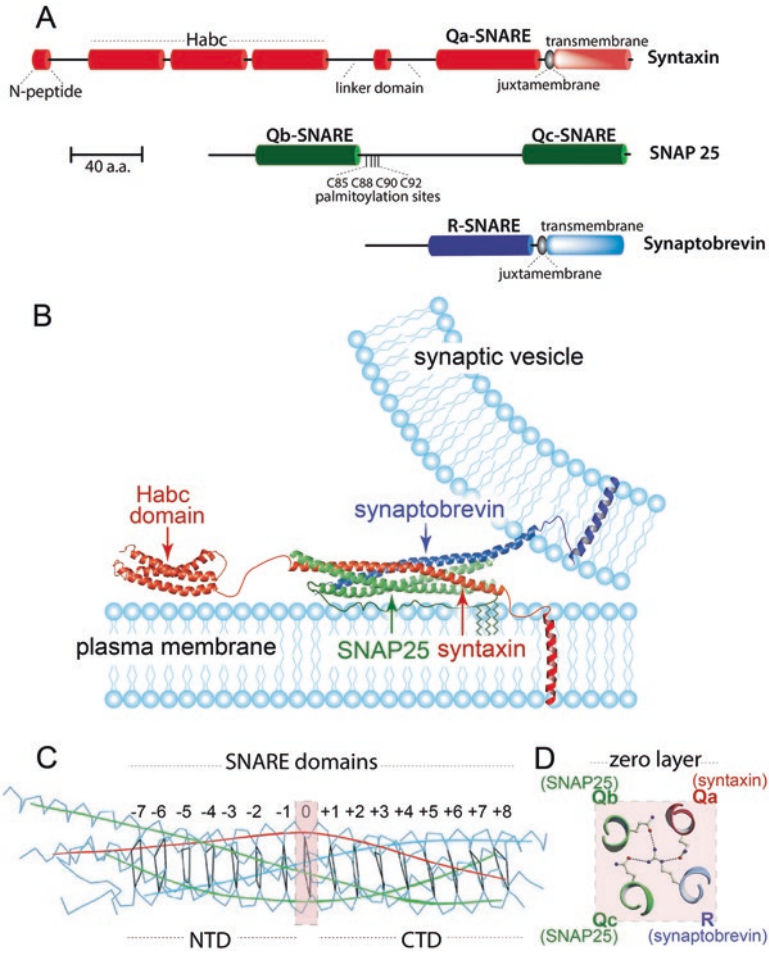

Fig. 1 Molecular description of the SNAREs. (**a**) *Synaptic SNARE proteins*. The SNARE motifs are 60–70 amino acid in length and form a four-helix bundle. Synaptobrevin (R-SNARE), and syntaxin (Qa) contribute one SNARE motif and SNAP25 contributes two SNARE motifs (Qb and Qc). Syntaxin contains an additional regulatory domain comprised of three alpha helices called the "Habc" domain. Syntaxin and synaptobrevin are tail-anchored transmembrane proteins while SNAP25 is attached to the membrane via palmitoylation of the linker region. (**b**) *Vesicle docking*. By assembling into a four-helix parallel bundle, the SNAREs bridge the gap between the two membranes destined to fuse. In the case of the neuronal SNAREs, syntaxin (red) and SNAP25 (green) are found on the plasma membrane and synaptobrevin (blue) is associated with the synaptic vesicle. The N-termini are at the left and the C-termini are at the right. (**c**) *SNARE complex*. The amino acids facing toward the center of this helix (denoted as layers −7 to +8) are largely hydrophobic in nature with the notable exception of the zero layer. (**d**) *Zero layer*. Charged residues are oriented toward the center of the helix: syntaxin contributes one glutamine (Qa), SNAP25 contributes two glutamines (Qb and Qc), and synaptobrevin contributes one arginine (R). (Illustration in (**b**) courtesy of Enfu Hui and Edwin R. Chapman. (**c**) is adapted from Ref. [40]. (**d**) is adapted from Ref. [456])

Proof that SNARE assembly could do active work on membranes came when Rothman's group demonstrated that the SNAREs alone could fuse membranes [38, 39]. Weber et al. incorporated SNAREs into vesicles composed of artificial lipid bilayers and demonstrated that donor vesicles containing synaptobrevin were capable of fusion with acceptor vesicles containing syntaxin and SNAP25 [38]. The SNAREs therefore function in the final steps of fusion and represent the minimal fusion machinery.

2 SNARE Structure

At each trafficking step in the secretory pathway, a unique SNARE complex is used [29]. The SNAREs can be classified functionally as vesicle SNAREs (v-SNAREs) or target SNAREs (t-SNAREs). The assembly of the SNARE complex bridges the vesicle and target membrane, forming what is known as a *trans* SNARE complex (Fig. 1b). The formation of the *trans* SNARE complex drives fusion.

Molecularly, the SNARE proteins are defined by the presence of a conserved 60–70 amino acid SNARE motif, and often also include an N-terminal regulatory domain and a C-terminal membrane anchor. The SNAREs comprise four different families that arose very early in eukaryotic evolution [29, 40–43]. They are defined at a molecular level as Qa-, Qb-, Qc-, and R-SNAREs based on the conserved residue at the center of the SNARE motif. The v-SNARE is usually an R-SNARE, and the t-SNAREs are usually the Qa-, Qb-, and Qc-SNAREs, although this arrangement is not universally true. Moreover, defining what is a "vesicle" and what is a "target" in fusion reactions is often meaningless; for example, transport vesicles fuse to generate a larger vesicle during homotypic fusion [44]. Therefore, we have adopted the Qabc and R nomenclature in this chapter.

The individual SNARE motifs are largely unstructured in solution [45–49], but when all four family members are mixed, the SNARE motifs come together to form a four-helix parallel bundle known as the core complex (Fig. 1b, c) [45, 50]. The SNARE complex is remarkably stable and can only be separated by boiling in the presence of sodium dodecyl sulfate (SDS) [51, 52]. The parallel orientation of the SNAREs [53], their assembly into a four-helix bundle [50], and the stability of the complex [52] led directly to the proposal that SNARE assembly might proceed by zippering from the N-termini to membrane-anchored C-termini.

Along the bore of the helix, the four alpha helical SNARE motifs are arranged in 16 layers of interacting residues (−7 to +8) (Fig. 1c). Fifteen of these layers (−7 to −1 and +1 to +8) consist of interacting hydrophobic residues; the "0" layer in the middle of the complex is formed by ionic interactions between an arginine (R) and three glutamines (Q) (Fig. 1c, d). The "0" layer residues are used to classify the four SNARE families as R- and Q- SNAREs, and can be further divided as Qa-, Qb-, and Qc-SNAREs based on their position in the four-helix core complex. In rare instances (Use1, Vti1, Sft1, and Bet1), an aspartate

(D) or a threonine (T) residue replaces one of the glutamines (Q) [29]. However, the presence of either aspartate or threonine is not conserved in all orthologs; for instance, Vti1 contains a D residue in yeast and mammals, but a Q residue in *Drosophila* and *C. elegans* [29]. The SNAREs used for synaptic vesicle exocytosis are synaptobrevin (R-SNARE, also called VAMP2), syntaxin 1a (Qa-SNARE, more generally known simply as syntaxin), and SNAP25 (which contains both the Qb- and Qc-SNARE motifs) (Fig. 1a) [1–4, 54].

In all SNARE-based fusion reactions, each of the two membranes destined to fuse must contain at least one SNARE with a transmembrane domain; otherwise, fusion will not occur [55]; membrane proximity alone is not sufficient to catalyze fusion [56]. When the transmembrane domain is truncated, mutated, or replaced with an artificial lipid anchor, fusion levels are reduced and, in most cases, no longer proceeds to complete membrane fusion [56–62]. Nevertheless, many of these perturbations still lead to a state in which lipids can exchange, suggesting hemifusion of the juxtaposed monolayers, or spontaneous flipping of individual lipids across the gap [58, 59, 61]. These findings are consistent with the energy requirements for fusion—the early steps of lipid exchange have been calculated to require less energy than the later stages of fusion pore formation and expansion [63].

In most fusion reactions, all four SNAREs possess a transmembrane domain and are encoded as individual proteins [29, 40, 43]. However, in the SNAREs used in post-Golgi trafficking, which include SNAP25, the Qb- and Qc-SNARE domains are coupled in a single protein lacking a transmembrane domain [64, 65]. SNAP25 is anchored via palmitoylation of cysteines in the linker connecting the two SNARE motifs (Fig. 1a). SNAP25 membrane association is not absolutely required for fusion, but mutation of the palmitoylated cysteines results in altered kinetics [66]. It is possible that fusing Qb- and Qc-SNAREs into a single polypeptide evolved to support the rapid calcium-triggered fusion of exocytic vesicles [66], although it is worth noting that several post-Golgi SNAREs including the yeast ortholog of SNAP25, Sec9p, also have coupled Qb- and Qc-SNAREs.

Many SNARE proteins have autonomously folding N-terminal regulatory domains [67]. All Qa-SNAREs, including the synaptic syntaxins, possess an Habc domain at the N-terminus [68]. Some R-SNAREs possess a Longin domain [69]. In rare instances, Qb- and Qc-SNAREs possess an Habc domain or other regulatory domains [70]. These exceptions have led to some confusion. Notoriously, “syntaxin 6” is not a Qa-SNARE; it is instead a Qc-SNARE by homology and behaves as a Qc-SNARE in complexes [71]. Syntaxin 6, despite its homology, was misnamed because it possesses an Habc domain like syntaxins/Qa-SNAREs [29, 70, 72]. Unfortunately, the name “syntaxin 6” stuck. At the synapse, syntaxin with its Habc domain is the only SNARE with an extended N-terminal domain (Fig. 1a, b). The R-SNARE homolog, synaptobrevin, does not have the evolutionarily more ancient Longin domain found in other R-SNAREs.

Both the Habc domain [73, 74] and the Longin domain [67, 75, 76] can fold over and occlude the SNARE motif of their respective proteins, although this mode of interaction is not conserved [67]. Adopting this occluded or "closed" state prevents the SNARE motif from prematurely interacting with other SNAREs [73, 76].

The simple model in which the main function of the Habc domain is to occlude SNARE interactions is not correct. First, in vivo, deletion of Habc dramatically decreases fusion rather than increasing fusion rates [77–80]. Second, Habc domains, as well as Longin domains, have been found on numerous non-SNARE proteins [75, 81, 82]. Third, the closed conformation is not generally conserved among syntaxin proteins [83]. Fourth, the Habc domain of syntaxin can function when SNARE motif and Habc domain are encoded as separate proteins (*in trans*) [80]. Fifth, the Habc is required to activate the SM protein Unc18 to initiate SNARE pairing, as described in detail below [84].

3 SNARE Genetic Redundancy

Perturbation of SNAREs in vivo usually fully eliminates a single trafficking step. However, in many cases the trafficking step was not completely eliminated. There are two possible explanations. First, it is possible that the SNAREs are not executing fusion—an unlikely interpretation given the wealth of data described above. Second, the SNAREs might be partially redundant. Evidence points to the latter interpretation. Knockout mice in synaptobrevin/VAMP2 were found to retain some synaptic activity in hippocampal neurons [17]. In chromaffin cells, this remnant activity could be attributed to the synaptobrevin paralog cellubrevin [85]. Redundancy can also explain the remaining fusion events in null mutants of n-Syb, the *Drosophila* equivalent of synaptobrevin. Syb, the *Drosophila* equivalent of cellubrevin, can functionally substitute for n-Syb when overexpressed in neurons [86]. Redundancy is also seen in the Q SNAREs. SNAP23, SNAP47, and SNAP24 can provide partial function when SNAP25 is absent [19, 87, 88]. Finally, redundancy might also explain the almost complete lack of phenotype in syntaxin 1a knockout mice [89], where it is likely that syntaxin 1b is sufficient to almost entirely replace syntaxin 1a [90]. These observations are supported by experiments in yeast where redundancy between SNAREs has also been conclusively demonstrated in numerous trafficking reactions [91–93]. By contrast, loss of syntaxin (*unc-64*) in *C. elegans* neurons results in a 500-fold reduction in neurotransmitter release with no apparent developmental defects [20]; UNC-64 is committed to synaptic vesicle fusion and is unlikely to have a redundant syntaxin, like in mice; nor is it involved in other cellular functions, like in flies [94]. In summary, the SNAREs largely function at single trafficking steps and are completely necessary for membrane fusion.

4 General Principles of SNARE-Based Trafficking

Before moving to synaptic vesicle fusion, we pause here to describe the four universally conserved steps in SNARE-based trafficking. Sequentially, they are as follows:

- Vesicles are tethered to target membrane
- SM proteins template SNARE assembly
- SNARE zippering drives fusion
- SNAREs are disassembled

4.1 Vesicle Tethering

Regulated trafficking requires a vesicle to first recognize and physically attach to its target—a step known as tethering (Box 1). Tethering is defined as a loose attachment to the membrane, and is visible by electron microscopy. Multisubunit

Box 1 Definitions: The World Turned Upside Down and Given a Good Shake

The nomenclature for steps in vesicle fusion relies on operational definitions. Unfortunately, the terms used by the synaptic community sometimes conflict with the nomenclature used by the yeast community, and have not always been used consistently by either the yeast or the synapse community.

Synaptic nomenclature:

- *Tethering*. Tethering is a morphological definition defined by electron microscopy. Often, physical tethers can be observed in electron micrographs as a darkly stained filament contacting the plasma and vesicle membranes [95]. More generally vesicles close to the plasma membrane, usually less than 20 nm, are considered tethered. Even vesicles that appear to touch the plasma membrane are considered tethered rather than docked, if they remain rounded and lack an electron dense contact site [95]. The precise molecular components of tethering are not yet known but are likely to include the active zone proteins: piccolo, bassoon, alpha-liprin, ELKS/CAST, RIM, RBP, Rab3/Rab27, and Unc13 [96]. Tethering is thought to be independent of SNAREs.
- *Docking*. Docked vesicles, also defined by ultrastructure, are vesicles in contact with the plasma membrane [95, 97–99]. In these fixed and stained samples, clearly distinguishable bilayers are not detected and the vesicle sometimes appears slightly flattened against the plasma membrane. This strict definition is backed up by genetic experiments indicating that docking requires SNAREs and Unc13.

- *Priming*. Priming is a molecular definition in which the SNARE proteins are engaged. SNARE engagement is required for morphologically defined docking, and for the electrophysiologically defined readily releasable vesicles. It is therefore likely that release-ready, docking, and priming define the same group of vesicles by different techniques [100]. What the precise molecular configuration is for primed vesicles is not yet known, although it must include SNAREs, synaptotagmin, complexin and probably includes Unc18 and Unc13. Moreover, priming is likely to include more than a single molecular state of SNARE assembly.
- *Readily releasable pool (RRP)*. The RRP is defined by electrophysiology. These are vesicles that can fuse, if they are exposed to calcium. A single action potential will not cause all release-ready vesicles to fuse due to the stochastic nature of calcium channel opening. However, the size of the pool can be determined by a succession of action potentials that exhaust the pool [101]. Vesicles in the readily releasable pool can also be driven to fuse in the absence of stimulation by applying hypertonic sucrose [102]. It is likely that hypertonic media dehydrates the cytoplasmic gap between the plasma and vesicle membranes of primed vesicles, stimulating SNARE-mediated fusion.

Yeast nomenclature:

- *Tethering*. In yeast, tethering is the first stage in membrane association. It is independent of SNARE proteins and dependent on small GTPases. Unlike the second stage—SNARE engagement—tethering is reversible. *Tethering* in yeast and synapses is roughly equivalent.
- *Docking*. Historically, docking in yeast refers to the entire process of membrane association of vesicles to their target membrane [103]. In the late 1990s, a reversible GTPase-dependent step that is independent of SNAREs was discovered and termed "*tethering*" [104]. Although docking is still sometimes used to refer to the entire process of membrane association, in recent years it has more often been applied to the *stable docking* step that follows tethering in which the SNAREs are engaged, thus falling in concert with the synaptic literature. A stable docked state, like that observed at synapses, is normally not observed in yeast, since SNARE engagement leads inexorably to fusion. However, stable docking can be observed in biochemical reconstitution experiments in which membrane fusion is prevented by reduced temperature.
- *Priming*. Priming in yeast nomenclature is defined as the separation of SNARE proteins by the ATPase NSF, so that the potential energy of unengaged SNAREs is now restored. This terminology is at odds with synaptic nomenclature and there is no equivalent terminology for SNARE separation in synapse nomenclature.

tethering complexes function at this step [105]. Loose membrane association proceeds to tight membrane association that is mediated by SNAREs. At synapses, this second stage is known as docking [20, 95]. Tethering factors and SNAREs serve overlapping roles in target recognition. Together their actions culminate in the initial N-terminal assembly of the SNAREs in diverse cellular trafficking events from yeast to vertebrates, from lysosomes to synaptic vesicles.

Multisubunit tethering complexes comprise a diverse collection of proteins. Broadly speaking, they can be divided into two general categories: CATCHR and non-CATCHR. CATCHR complexes (Complexes Associated with Tethering Containing Helical Rods) include Dsl1, COG, GARP, and exocyst. Non-CATCHR complexes include TRAPP I, II, III, HOPS, and CORVET. Despite the lack of sequence conservation, multisubunit tethering complexes share architectural features: they have common structural elements and subunit organization [106–108]. Some of these complexes have been verified to act as tethers, that is, they can physically link vesicle and target membranes; others may act indirectly in tethering by regulating SNARE assembly [109]. Most likely, multisubunit tethering complexes serve both functions: they physically link the vesicle to the target, and also regulate SNARE assembly.

Overlapping roles for factors mediating tethering and SNARE assembly have been observed in yeast [110–115]. For instance, *sec35,* a tethering protein for Golgi trafficking, can be partially bypassed by overexpression of the relevant SNARE proteins [113]. Similarly, mutations in the tethering complex for plasma membrane fusion can be bypassed by SNARE overexpression [111, 114]. Suppression is not bidirectional—SNARE overexpression can bypass tethering factors, but tethering factors cannot bypass SNAREs—demonstrating that SNAREs act downstream of tethering factors [115].

At synapses, the MUN domain proteins Unc13 and CAPS tether synaptic vesicles and dense core vesicles to fusion sites [5, 20, 100, 108, 116–120]. Structurally, the MUN domain resembles the CATCHR family used in trafficking to and from the Golgi [106, 117, 121, 122]. The C2 domains that flank the MUN domain bind to the synaptic vesicle and the plasma membrane, thereby bridging the two membranes destined to fuse [118]. Additionally, Unc13 plays an active role in SNARE assembly [20, 123–128]. Unc13 is thus a membrane tether and a regulator of SNARE-dependent docking.

At each trafficking step along the secretory pathway a unique SNARE complex is used, leading to the model that SNAREs *alone* could direct target specificity [28, 129–131]. This simple model is not correct. In vivo, tethering complexes bring vesicles to the correct fusion sites; in their absence, vesicles do not successfully reach their targets.

Nevertheless, SNARE compatibility is still an essential component for directing fusion to a specific target. When inserted in artificial membranes, SNAREs exhibit specificity in catalyzing fusion reactions [129, 130]. Specificity can also be seen in vivo; after cleavage of SNAP25 in PC12 cells, secretion could only be rescued by

SNAP25 itself and not by other SNAP25 homologs [131]. Finally, the removal of SNARE proteins results in defects in respective membrane attachment [20, 84, 95]. Despite early evidence to the contrary [13, 14, 16, 132], it is now clear that SNAREs can mediate the specificity and physical attachment of vesicles to their target membrane [20, 84, 95, 133–135]. In vivo, a combination of tethering factors and regulated SNAREs assembly is necessary to precisely dock synaptic vesicles at the active zone [44, 136–141]. The partially overlapping functions of tethering factors and SNAREs is needed to achieve the high level of spatial fidelity seen in vesicle fusion.

4.2 SM Proteins Template SNARE Assembly

SM proteins are conserved in all SNARE-based membrane fusion events [142]. They are the fifth Beatle to the four SNAREs—SNARE assembly cannot be considered without them. The importance of the SM proteins is underscored by their presence in every known SNARE-mediated membrane fusion reaction [143, 144], and the dramatic phenotypes that result from their absence [145–149]. In the case of synaptic vesicle fusion in mice, for instance, the removal of Munc18 results in a defect in synaptic vesicle fusion that is as profound as the removal of the SNAREs themselves [145].

SM proteins all have a common structure [74, 150–160]. Roughly speaking, they have a globular body with a protruding hairpin structure, domain 3a, that can be furled or unfurled [74, 154, 161–164] (see Fig. 4). Between the globular body and domain 3a lies a prominent groove. As such, the full structure looks like a mitten—the globular body representing the palm, and domain 3a the thumb. The first structure of an SM protein was of Unc18 gripping syntaxin in the closed state. Interestingly, this structure did not represent a conserved binding mode between SM proteins and syntaxin, but nevertheless, dominated thinking about the role of Unc18 in the years that followed.

The importance of SM proteins has been clear for many years, but understanding the role for SM proteins in SNARE assembly has been confounded by the numerous binding modes between SNAREs and SM proteins [165]. It is now clear that the different binding modes exist to allow SM proteins to regulate multiple steps during the trafficking and assembly of SNAREs. By binding SNAREs, SM proteins (1) block inappropriate SNARE interactions, (2) transport syntaxin, (3) template correct SNARE interactions, and (4) protect SNARE disassembly by NSF. Templating represents the universally conserved role of SM proteins. The relative importance of the other roles depends on the specific requirements of the membrane trafficking step. In the case of synaptic transmission, the SM protein Unc18 must get syntaxin to the synapse and prevent it from prematurely interacting with other SNAREs.

4.3 SNARE Zippering Drives Fusion

SNARE assembly proceeds via zippering from the N-termini to C-termini. The concept that SNARE zippering could drive membrane fusion came from three key observations: (1) the SNARE complex is remarkably stable [52], (2) the SNAREs assemble in a parallel orientation [53] and (3) assembled SNAREs form a coiled-coil structure [50]. The nucleation of the SNARE complex at the free N-termini followed by progressive assembly of the complex would pull the vesicle and the plasma membranes together to drive fusion (Fig. 1b) [53, 166–177].

The actual evidence for zippering came initially from two complementary experiments. First, biochemical and structural studies demonstrated that the membrane-proximal domain of syntaxin becomes sequentially more ordered upon binding synaptobrevin in a directed N- to C-terminal fashion [169, 178–180]. The temperatures for assembly and disassembly of SNARE complex differ by as much as 10 °C. Thus, assembly and dissociation follow different reaction pathways. Temperature hysteresis is evidence of a kinetic barrier between folded and unfolded states [51, 172, 173]. Mutations in the N-terminal hydrophobic core of the SNARE complex selectively slowed SNARE assembly while those in the C-termini did not [169, 181], suggesting that SNARE assembly is nucleated at the N-termini and that loose SNARE complexes might be a stable intermediate [182]. The second line of evidence for zippering came from in vivo disruption studies using clostridial toxins, antibodies directed toward the SNARE motifs, and mutations in the hydrophobic core of the SNARE complex [166, 168, 169, 183, 184]. These studies demonstrated that the N-termini of SNAREs become resistant to cleavage or antibody block at early stages of SNARE assembly, while C-termini are only resistant to disruptions at late stages.

Recent advances in technology, such as optical tweezers, have confirmed that SNARE assembly proceeds by zippering [171, 173–177]. Zippering proceeds in three distinct steps. Initial zippering takes place at the N-termini of the SNARE motif, this is followed by a pause at the half-zippered state, then zippering proceeds to the C-termini [173]. Zippering is an intrinsic property of SNARE proteins [171, 185] and does not require additional factors [172, 173].

4.4 SNARE Disassembly

After the two membranes have merged, the SNARE complex is located in a single membrane and is referred to as a *cis* SNARE complex. Repeated rounds of vesicle fusion require SNARE disassembly. NSF and SNAPs disassemble the *cis* SNARE complex, allowing the SNAREs to be repartitioned to their appropriate compartments. NSF uses ATP to disassemble the SNAREs, and much like a battery, the energy put into the system is stored in the monomeric SNARE proteins. This energy will be released during SNARE winding to fuse membranes. Together, NSF and the SNAPs are able to disassemble all SNARE complexes [186–189]. The ATPase NSF itself

does not directly bind SNAREs, instead it binds SNAREs through the action of the SNAPs [187, 190]. SNAP proteins (Soluble *N*-ethylmaleimide-Sensitive Factor Attachment Protein) are not related to the SNARE protein SNAP25. There are three SNAP proteins: αSNAP, βSNAP, and γSNAP [27]. αSNAP and βSNAP are closely related and became duplicated in the vertebrate lineage. αSNAP is ubiquitous, and βSNAP is brain specific [191]; they act together in regulated exocytosis in neuronal cells [192–194]. γSNAP is found in all phyla and is dedicated to SNAREs involved in endosome trafficking [195]. The SNAPs bind to the surface of the *cis* SNAREs around the central zero layer, which contains the conserved Q and R residues [190, 196]; although it is not clear whether these residues are important for disassembly [197, 198]. NSF does not disassemble SNAREs by pulling an unwound SNARE through the pore of the ATPase. Current models suggest that dislocation of the N-terminus of SNAP25 by the membrane-proximal ring of NSF, or reverse torque applied to the complex by the SNAP proteins, could cause the complex to disassemble [187, 188, 199].

NSF and αSNAP disassemble *cis* SNAREs and police the synapse for incorrectly assembled SNAREs that may have wandered astray during assembly. αSNAP and NSF can disassemble numerous off-pathway SNARE complexes, including non-cognate, antiparallel, and non-stoichiometric complexes [187]. Indeed, NSF can also disassemble productive *trans* SNARE complexes. In a physiological setting, the on-pathway SNAREs are protected from the action of NSF by Unc18, Unc13, complexin, and synatotagmin [200–202]. Disassembled syntaxin is also rapidly bound up by Unc18, the starting point for a new round of SNARE assembly and the release of another synaptic vesicle. Thus, NSF serves not only to recycle *cis* SNAREs but also as a quality control mechanism during SNARE assembly.

5 Assembling Snares at Synapses

Cycles of SNARE assembly and disassembly underlie rounds of vesicle fusion: assembly leads to vesicle fusion; disassembly prepares the SNAREs for another round (Fig. 2). At synapses, SNARE interactions must be tightly regulated to ensure the spatial and temporal fidelity of membrane fusion. The process of SNARE-mediated synaptic vesicle fusion can be divided into seven steps:

- *Transport: Unc18 chaperones syntaxin during trafficking*
- *Tethering: Unc13 tethers synaptic vesicles*
- *SNARE pairing: UNC-18 templates SNARE assembly*
- *Priming: synaptotagmin and complexin pause SNARE winding at the half-zippered state*
- *Disinhibition: calcium binds synaptotagmin and unleashes SNARE zippering*
- *Fusion: zippering of the SNARE C-termini transfers energy to the transmembrane domains and drives fusion*
- *Disassembly: NSF and αSNAP separate the SNARE complex*

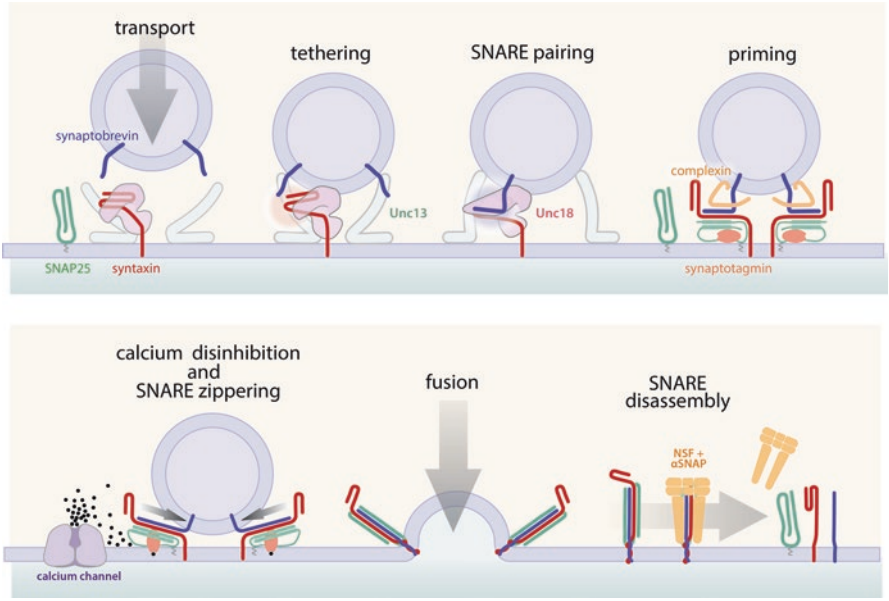

Fig. 2 Overview of SNARE assembly and membrane fusion at synapses. *Transport*, synaptobrevin is transported to the synapse by kinesin on synaptic vesicle precursors. Syntaxin and SNAP25 are broadly localized in axons. *Tethering*, the synaptic vesicle is recruited to a release site by Unc13 and syntaxin is converted to the open state. *SNARE pairing*, the open state of syntaxin stimulates Unc18 to template and proofread syntaxin and synaptobrevin pairing. *Priming*, Unc13 and Unc18 recruit SNAP25, and synaptotagmin and complexin hold the vesicle in the paused, half-zippered state. (Only the C2B domain of synaptotagmin is shown). *Calcium*, membrane depolarization opens calcium channels, calcium-binding releases the synaptotagmin block. *Fusion*, SNARE winding pulls the membranes together and creates a fusion pore. *SNARE diassembly*, alpha-SNAP binds the complex and the ATPase NSF separates the SNAREs

In the remainder of this chapter, we will go through each of these steps, detailing the proteins and membrane rearrangements that take place.

5.1 Transport and Trafficking SNAREs

Upon exit from the Golgi, synaptobrevin is sorted into synaptic vesicle precursors and transported by kinesin to the synapse within BLOC-One-Related Complexes (BORC) [203–213]. Syntaxin is transported on vesicles by the kinesin adaptor protein Fez1/UNC-76 [214]. The Qbc-SNARE SNAP25 lacks a transmembrane domain but is palmitoylated in the Golgi and is transported to the plasma membrane by the secretory pathway [215–217], perhaps in association with kinesin-1 [218]. The Qbc-SNARE SNAP25 is broadly localized to the plasma membrane of

the axon where it can be found in clusters [217, 219]. Syntaxin is also not specifically localized at synapses but rather decorates the axoplasm uniformly or in broad clusters [217, 219–222] and is transported there during growth cone extension [223, 224].

5.1.1 Unc18 Chaperones Syntaxin to the Axoplasm

Promiscuous assembly is an intrinsic property of SNARE proteins, and this presents a problem during transport. SNAREs can assemble with non-cognate SNAREs, they can assemble with the wrong stoichiometry, and they can assemble in antiparallel configurations [46, 201, 225–229]. SNAREs are sticky proteins and many identified binding partners are likely to be irrelevant or artifactual. On the other hand, legitimate binding targets include non-SNARE proteins that regulate trafficking or fusion [230]. It is clear these errant teenagers require a chaperone. At the cotillion of SNARE assembly, SM proteins ensure that only productive SNARE complexes are formed.

The first step is to simply exit the Golgi and to get to the plasma membrane of the axon without interacting with other SNARE proteins. In neurons, the SM protein Unc18 (UNC-18/ Munc18/nSec1) binds tightly to closed syntaxin [231] and transports syntaxin to the plasma membrane [232]. Unc18 binds syntaxin in the closed conformation with the Habc domain folded over the SNARE motif (see Fig. 4b) [74]. This conformation prevents premature interactions with other SNARE proteins [74, 231–236].

The mammalian homolog Munc18-1 binds syntaxin with a Kd of ~1–5 nM [74, 159, 235, 237–239]. This high-affinity binding led to the proposal that Unc18 inhibits vesicle fusion [240–242]. However, the genetic evidence is not consistent with this model. Moreover, this mode of binding is not universally conserved; the binding of Unc18 to closed syntaxin monomers appears to be a unique adaptation for synaptic vesicle release [83, 157, 243–245]. The strength of this interaction may reflect the importance of trafficking syntaxin and Unc18 in neurons. In the absence of Unc18, syntaxin accumulates in the soma and is unstable [78, 79, 84, 232–236, 246–248]. For a protein, the distance between cell body and synapse can be immense, and protecting syntaxin from inappropriate interactions during transport is likely to be particularly important.

By contrast, the distance that Qa-SNAREs must be transported in yeast is comparatively short. Perhaps as a consequence, the binding to closed syntaxin monomers may not be a priority. When SM proteins do interact with Qa monomers, it is often binding that promotes open syntaxin, rather than stabilizing the closed form [153, 249, 250]. For instance, Vps45, the SM protein used in yeast Golgi fusion binds to its syntaxin partner Tlg2 in an open conformation [153]. The contrasting priorities for transport are likely to explain the difficulties for the SNARE community in arriving at a consensus for SM protein function.

5.2 *Unc13 Tethers Synaptic Vesicles and Initiates SNARE Assembly*

Synaptic vesicles in the reserve pool feed vesicles to fusion sites in the active zone. These release sites are tightly coupled to calcium channels [251], and are organized by Unc13 [252, 253]. The C1-C2B-MUN-C2C fragment of Unc13 is conserved in all isoforms and is responsible for tethering vesicles and activating syntaxin [254] (Fig. 3a). The Unc13 C2C domain binds to lipids and to synaptobrevin on a synaptic vesicle [118, 255–257]; the C2B domain binds negatively charged lipids in the

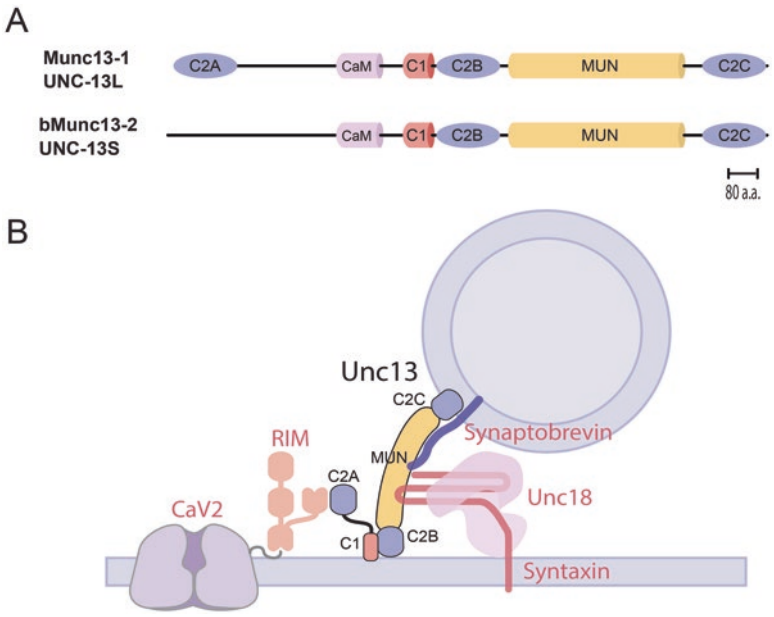

Fig. 3 Unc13 tethers synaptic vesicles and activates syntaxin. (**a**) *Domain architecture of Unc13*. All Unc13 isoforms contain a conserved C1-C2B-MUN-C2C fragment that is responsible for tethering vesicles and activating syntaxin. The C2C domain binds synaptic vesicles, the C2B domain binds the plasma membrane. C1 bind diacylglycerol (DAG) and modulates Unc13 activity. The MUN domain binds syntaxin. Along with this C-terminal fragment, Unc13 isoforms contain variable N-terminal extensions that are responsible for the organization of active zones, particularly the localization of calcium channels. The C2A domain of Unc13 binds Rab3-interacting molecule (RIM). Illustrated are the two predominant vertebrate Unc13 isoforms present in hippocampal neurons: Munc13-1 and bMunc13-2. *C. elegans* and *Drosophila* have two isoforms with similar architecture to Munc13-1 and bMunc13-2 [252, 253]. The N-termini are at the left and the C-termini are at the right. (**b**) *Unc13 organizes release sites*. Unc13 binds synaptic vesicle directly via C2C domains and indirectly by binding synaptobrevin. C2B domains bind the plasma membrane, tethering synaptic vesicles. Vesicle tethering is localized to calcium channels via the variable N-terminal of Unc13 that binds to RIM, which in turn binds the calcium channel CaV2. Interactions between the MUN domain and syntaxin activate syntaxin allowing it to form the 4-helix core SNARE complex

plasma membrane (Fig. 3b). Together, the C2C and C2B domains allow Unc13 to bridge vesicles and plasma membranes [118], likely keeping them ~20 nm apart [256]. In vivo, the absence of Unc13 eliminates tethering and docking [20, 95, 258]. Along with bridging synaptic vesicle and plasma membranes, Unc13 guides synaptic vesicles to calcium channels via variable N-terminal domains. Specifically, the C2A domain, present in some isoforms, is linked to a calcium channel via the active zone protein RIM [140, 259–262].

Unc13 also serves an active role in SNARE assembly: Unc13 converts syntaxin to an open state to promote formation of the SNARE complex [20, 123–127, 263]. The closed, inhibited, state of syntaxin is a specialized property of SNAREs used in exocytosis. Unc13 likely opens syntaxin by interacting with the linker domain separating the syntaxin SNARE motif from the regulatory Habc and N-peptide [264] (Fig. 1a). Mutations in the linker domain, the so called "LE" mutations, lead syntaxin to adopt an open conformation [73]. In the absence of Unc13, synaptic vesicle fusion is abolished [265–268]. However, the constitutively open form of syntaxin can partially restore fusion [20, 123, 269] and can fully rescue *unc-13* docking defects [20]. The discrepancy between full rescue of docked vesicles and partial rescue of fusion hints at potential roles for Unc13 downstream of docking. We will explore those in the coming sections.

Thus, Unc13 stands ready at release sites, cradling syntaxin bound tightly by Unc18. Recruitment of a synaptic vesicle from the reserve pool by the C2C domain signals to the MUN domain to convert syntaxin to the open state and offer it to synaptobrevin.

5.3 Unc18 Templates SNARE Assembly

In the closed state of syntaxin, Unc18 keeps the SNARE motif broken up into several smaller helices that are trapped in place by binding between the Habc domain and Unc18 [74] (Fig. 3a). This structure is incompatible with SNARE assembly and, it was theorized, would need to be dismantled for syntaxin to adopt the "open" form that would initiate SNARE assembly [73, 74].

Experimentally, however, Unc18 can bind both open and closed syntaxin [152, 236, 270]. Furthermore, the unfolding energies for Unc18 bound to closed syntaxin and bound to open syntaxin are 7.2 and 2.6 k_BT, respectively [271]. This implies that only 4.6 k_BT is required to open syntaxin, a figure far less than the ~22 k_BT that has been calculated to wrestle Unc18 free of syntaxin [159].

The opening of syntaxin does not involve the removal of Unc18, as was once envisioned; rather Unc18 remains associated with syntaxin. The structure of Unc18 bound to open syntaxin can be predicted from the structure of Vps45, the SM protein involved in vesicle fusion in the Golgi from yeast, bound to its syntaxin partner Tlg2 in an open conformation [153]. In the threaded structure, Unc18 remains attached to syntaxin via continued association with the Habc domain, the N-peptide of syntaxin bound to domain-1 of Unc18, and possibly via residues at the bottom of

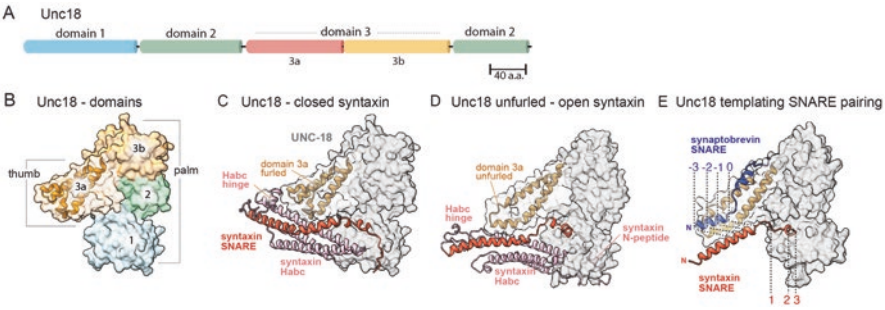

Fig. 4 SNARE templating by Unc18. (**a**) *Domain architecture of Unc18*. Unc18 can be divided into four domains; domain 3 is further divided into domain 3a and 3b. The N-termini are at the left and the C-termini are at the right. (**b**) *Unc18 structure*. Unc18 folds into a structure that roughly resembles a mitten. Domain 3a forms the thumb of the mitten; the other domains form the palm. (**c–e**) *Potential steps for nucleating pairing of syntaxin and synaptobrevin by Unc18, as predicted from molecular structures*. (**c**) *Syntaxin closed state*. Syntaxin resides on the plasma membrane and is bound by Unc18 in the closed state. The Habc domain occludes the N-terminal half of the SNARE motif. Unc18 domain 1 binds the C-terminal half of the SNARE motif. (**d**) *Open syntaxin unfurls Unc18*. Unc13 recruits a synaptic vesicle to a release site and acts on the hinge domain to open syntaxin. The Habc domain transduces this conformational change to Unc18, and a segment from domain 3a in Unc18 flips out to create the "unfurled" or "open" conformation. (**e**) *SNARE pairing*. The unfurled domain of Unc18 can recruit synaptobrevin and aligns the N-termini of the syntaxin and synaptobrevin SNARE motifs. For **a**, UNC-18 and syntaxin/UNC-64a sequences from *C. elegans* were threaded onto the crystal structure of Munc18-1 bound to closed syntaxin-1 (PDB ID: 3C98). For **b**, the sequence of UNC-18 was threaded onto Vps45p and UNC-64a was threaded onto Tlg2p from the yeast Vps45-Tlg2 complex (PDB ID: 6XM1). For **c**, the UNC-18 sequence was threaded onto Vps33p, UNC-64a was threaded onto Vam3p; SNB-1 was threaded onto Nyv1p from the yeast Vps33-Vam3-Nyv1 complex (PDB ID: 5BUZ, 5BV0). The unstructured and therefore missing sequences in Munc18, syntaxin and Nyv1 crystal structures were assigned unstructured regions by the threading program. Homology modeling was performed using SWISS-MODEL [457], and visualization was performed using ChimeraX [458]

domain 3a that interact with the SNARE motif of syntaxin [263] (Fig. 4b). The relative importance of these binding interfaces is still in dispute. Some studies indicate that removal of the N-peptide does not perturb SNARE assembly and fusion [78, 84, 272, 273], whereas others indicate the opposite [79, 274–276]. Much like the open form of syntaxin, the binding mode of SM proteins to the syntaxin N-terminal regulatory domain is not conserved: some SM proteins favor N-peptide association whereas others favor Habc interactions [277]. Irrespective of the exact mode of binding, it is clear that all SM proteins remain attached to SNAREs to template their assembly. In neurons, Unc13 catalyzes the transition between closed and open syntaxin.

In neurons, the tight binding between Unc18 and closed syntaxin monomers masked a weaker but far more central role for the SM proteins: templating SNARE assembly. Templating was discovered in yeast, where tight binding between SM proteins and syntaxin monomers is not seen. The transient templating interaction between SM proteins and SNAREs very likely represents the evolutionarily conserved central function of SM proteins [84, 278].

The first hints at a proofreading role for SM proteins came from studies of the SM protein, Sly1, and its cognate syntaxin partner, Sed5. In the absence of Sly1, Sed5 formed complexes with numerous non-cognate SNAREs [274, 279]. Peng and Gallwitz proposed that the SM protein, Sly1p, might be proofreading the assembly of the Qa-SNARE Sed5 and the R-SNARE Bet1 [279, 280]. They were remarkably prescient.

In 2007, the labs of Tom Melia and Jim Rothman showed that in a liposome fusion assay the SNAREs were activated by their cognate SM proteins [281]. The strong stimulation of fusion that they observed was dependent on direct contact between the SM proteins and the Qa- and R-SNAREs. They suggested that SM proteins proofread SNARE assembly.

In 2015, the lab of Fred Hughson provided the first visual evidence for the proofreading step when they crystalized two complexes: (1) the SM protein Vps33 with the Qa-SNARE Vam3, and (2) Vps33 with the R-SNARE Nyv1 [151]. A composite of the two structures—experimentally supported by the presence of the tripartite structure in size exclusion chromatography—showed that Vps33 provides a template for Qa- and R-SNARE assembly [151]. In this structure, the C-terminal halves of the Vam3 and Nyv1 SNARE motifs are splayed apart on the surface of the SM protein [151]. By contrast, the N-terminal half of the SNARE motifs are aligned along an extended 3a domain of Vps33. The structure looks like a half-zippered SNARE complex, with the N-terminal domains aligned [151].

The extension of domain 3a, seen in Baker et al., 2015, is a conserved feature of SM proteins and is now understood to initiate SNARE templating [84, 161–164]. Like Vps33, domain 3a of neuronal Munc18-1 transitions from a compact furled loop (closed state) to an extended helical structure (open state) [154]. The extended domain 3a of Unc18 opens the platform for the assembly of a half-zippered syntaxin-synaptobrevin SNARE complex—the C-terminal half of the SNARE motif lies in the palm, the N-terminal half along the extended thumb [151]. The tripartite templating complex, consisting of SM protein and Qa- and R-SNAREs, has been observed in vitro [151], and in vivo experiments support its functional importance [84, 282]. Optical trap experiments and site-directed mutagenesis indicate that neuronal Munc18-1 shares the same templating functions as Vps33 [271].

We can create a potential structural pathway for Unc18 function by threading the sequence of UNC-18 onto the structures of SM proteins in various binding modes (Fig. 4c–e). From transport through the initiation of SNARE assembly, Unc18 binds syntaxin in the closed state (Fig. 4c) [78, 79, 84, 232–236, 246–248, 283, 284]. At the active zone, vesicles are tethered and presented to the Unc18-syntaxin complex by Unc13. Unc13 converts syntaxin to the open state. Through an unknown mechanism, likely involving the Habc domain [84], the domain 3a of Unc18 becomes unfurled (Fig. 4d) exposing a platform for syntaxin and synaptobrevin SNARE motifs, templating their assembly (Fig. 4e).

It is important to note that VPS33 does not interact with the Qa-SNARE in a closed state. It is likely that the high-affinity binding of Unc18 to closed syntaxin blinded us to this essential function of Unc18. The templating complex has an unfolding free energy of 5.2 k_BT and a lifetime of 1.4 seconds. The transient nature

of the complex is consistent with its role in proofreading, and probably helps explain its elusiveness to experimentalists. SNARE assembly needs to only briefly pause on the way to fusion. A templating complex that is too stable will never transition to fusion and one that is not stable enough will not provide the time for proper proofreading. To stabilize the neuronal templating complex for structural studies, Jose Rizo's lab crosslinked an open form of Syntaxin 1A with synaptobrevin and crystalized it with the open form of Munc18-1 [285]. The structure they solved looks remarkably like the tripartite, templating, Vps33-Vam3-Nyv1 complex [151, 285]. A strong case for the universal templating function of SM proteins can be made from these in vitro data.

The physiological importance of templating in vivo has been tested by engineering directed mutants based on templating structures. Mutation of the residues in Munc-18-1, which interact with syntaxin and synaptobrevin, seriously impairs synaptic vesicle release [282]. More strikingly, the yeast SM protein, Sec1, which normally provides no rescue to worm synaptic vesicle release, can rescue synaptic transmission when it is engineered to template worm SNAREs, if it can also interact with the Habc domain of syntaxin [84]. The requirement for the Habc domain is consistent with the known in vitro stabilizing role for this domain in the templating complex [143, 271, 286]. After years of searching for the enigmatic, conserved, positive function of SM proteins, these in vitro and in vivo studies have finally provided the answer: SNARE templating.

5.3.1 Revisiting the t-SNARE Acceptor Complex

The templating complex—a 1:1:1 complex between an SM protein and the Qa- and R-SNARE—does not include the Qb- and Qc-SNARE motifs. This realization prompted a reassessment of the "acceptor t-SNARE complex" model in which a 1:1 complex between syntaxin and SNAP25 represents the first stage in SNARE assembly. The "acceptor complex" had become the default starting point for effectively all in vitro SNARE assembly reactions [178, 181, 228]. But is it the physiologically relevant starting point in vivo?

The dynamics of the acceptor complex are not optimal. In liposome fusion assays, the acceptor complex does speed up the assembly of the core complex [179]. The binding of the acceptor complex to synaptobrevin can be quite fast, ranging from rate constants of 6×10^3 to 5×10^5 M^{-1} s^{-1} [179, 287], compared to the minutes to hours that individually mixed SNAREs require to assemble [181]. However, even with the acceptor complex, fusion rates do not approach the rates seen in vivo. In assays where the rate of fusion is more closely mimicked, the acceptor complex did not speed fusion [288], and indeed in some cases could result in a docked state that would persist for as long as 30 minutes [289].

The acceptor complex also readily misfolds [290, 291]. It will rapidly incorporate another syntaxin molecule to form a dead-end Qaabc four-helix complex [179, 292, 293]. In addition, the acceptor complex will readily assemble with tomosyn [294–298], a negative regulator of fusion [299–302]. Thus, in vivo, the acceptor

complex represents, at best, a problematic on-pathway starting point for SNARE assembly. Instead, it is a highly reactive complex that can sometimes proceed to productive fusion, but more often gets shunted off to non-productive end points.

In vivo, Unc18 binding to syntaxin monomers shields syntaxin from incorporation into "acceptor" complexes. Complexes that escape the protection of Unc18 are likely to be quickly dealt with by αSNAP and NSF, that together can disassemble a wide range of SNARE complexes [200, 202, 303, 304]. There is still active debate about the assembly order of the SNAREs [305, 306]. But SNAP25 appears to have gone from the first SNARE to enter the complex to the last [255, 271, 307, 308]. Instead of the acceptor complex, it is probable the Unc18-syntaxin complex represents the true physiological starting point for SNARE assembly [283] (Fig. 2).

At synapses, it is possible that Unc18 templating is aided by Unc13 through a yet to be unraveled mechanism [309]. In an in vitro assay, absence of either Unc18 or Unc13 causes an increase in antiparallel SNARE complexes [143, 225]. When both proteins are lacking as many as 40% of the complexes are assembled in antiparallel orientation [225]. The central MUN domain of Unc13 is known to bind syntaxin, SNAP-25, and synaptobrevin [124, 128, 255, 264, 307] and may therefore help incorporate SNAP25 into SM templated syntaxin-synaptobrevin pairs. However, when and how SNAP25 is recruited to prime vesicles for fusion is not known.

5.4 Synaptotagmin and Complexin Hold the SNAREs in a Half-Zippered State

After the SNAREs have been aligned and the complex nucleated at their N-termini, SNARE assembly pauses at a half-zippered state [173, 182]. This pause is an intrinsic property of SNAREs [173, 182]—it occurs in the absence of other proteins—and is likely the result of two factors. First, the repulsive forces of closely apposed membranes maintain the half-zippered state [173]. Second, the conserved zero layer residues may disrupt zippering and leave the C-termini splayed open momentarily [166, 310].

Neurons have exploited this intrinsic pause in SNARE zippering to link calcium influx to rapid and synchronous membrane fusion. All membrane fusion events are facilitated by calcium. Facilitation can be indirect, for example by binding proteins such as calmodulin [311]. Alternatively, it can be direct: calcium is a divalent cation that can act directly on membranes, for example by neutralizing the negative charges of phosphatidylinositol 4,5-bisphosphate (PIP2) [312–317]. But only in neurons is calcium exquisitely tied to triggering SNARE-mediated fusion. Two changes make this possible: (1) synaptotagmin and complexin stabilize the half-zippered state and are disinhibited by calcium, and (2) voltage-gated calcium channels are tightly localized to synaptic vesicle fusion sites, minimizing calcium diffusion [251, 318]. Together these factors allow for the delay between the elevation of cytosolic calcium and the postsynaptic response to be as short as 60–200 μs [319].

Broadly speaking, complexin and synaptotagmin stabilize the half-zippered state; calcium relieves this inhibition (Fig. 5). Complexin and synaptotagmin are both brakes and facilitators of fusion, they resemble the anchor escape mechanism that synchronizes pendulum clocks. Calcium binding to synaptotagmin releases the catch and SNARE zippering rapidly propagates and pulls the membranes together [320–323]. We are only just beginning to understand the mechanism of stabilization. However, we do not understand the mechanism of disinhibition. The rapid structural changes that underlie calcium sensing represent one of the great remaining mysteries in synaptic transmission.

5.4.1 Synaptotagmin

Synaptotagmin is an integral membrane protein of the synaptic vesicle composed of tandem calcium-binding C2 domains: C2A and C2B [324–328] (Fig. 5a). Null mutants in synaptotagmin dramatically decrease calcium-triggered, evoked synaptic vesicle release with a concomitant increase in spontaneous fusion [327, 329]. Thus, synaptotagmin acts as a brake on spontaneous fusion, pushing the vesicle release machinery into a state that is preferentially geared to calcium triggering. Mutations that alter calcium-binding affinity of synaptotagmin-1 lead to parallel change in the calcium sensitivity of synaptic vesicle release [328, 330]. Calcium stimulated interactions between synaptotagmin and the phospholipid, PIP2, have a Kd of 10 μM calcium [326], which closely matches the EC50 measured for calcium to trigger vesicle fusion [331]. Synaptotagmin is therefore the major calcium sensor for vesicle fusion, and phospholipid binding is key to its function.

The C2 domains of synaptotagmin interact with SNAREs and membranes in both a calcium-dependent and a calcium-independent manner [332–336]. C2B mutations more severely impair evoked release than C2A mutations [337–339]; however, it is likely that both calcium-binding domains coordinate during vesicle fusion through as of yet unknown mechanisms [335, 340–342]. Calcium causes the C2B binding domain to toggle between two different membrane-binding conformations: In the absence of calcium, positively charged lysine residues on the "ventral" surface of the C2B domain bind acidic phospholipids and hold the C2B domain in a horizontal configuration [343–345] (Fig. 5b). In the presence of calcium, the hydrophobic lips that surround the calcium-binding pocket penetrate the phosphatidylinositol membrane and are likely to rotate the C2 domain into a vertical orientation [346, 347] (Fig. 5c).

Fig. 5 (continued) The accessory helix (AH) of complexin holds the C-termini of SNAP25 Qc (green) and synaptobrevin away from the other SNARE helices. The synaptotagmin C2B domain (gold) sits under the SNARE complex preventing zippering beyond the 0 layer, and interacts with the plasma membrane. (**c**) *Fusing vesicle*. Upon calcium binding (black dots), the calcium-binding loops of the synaptotagmin C2B domain rotate into the plasma membrane, driving synaptotagmin out from under the SNAREs. This removes the block to fusion, allowing the SNAREs to fully zipper leading to synaptic vesicle fusion

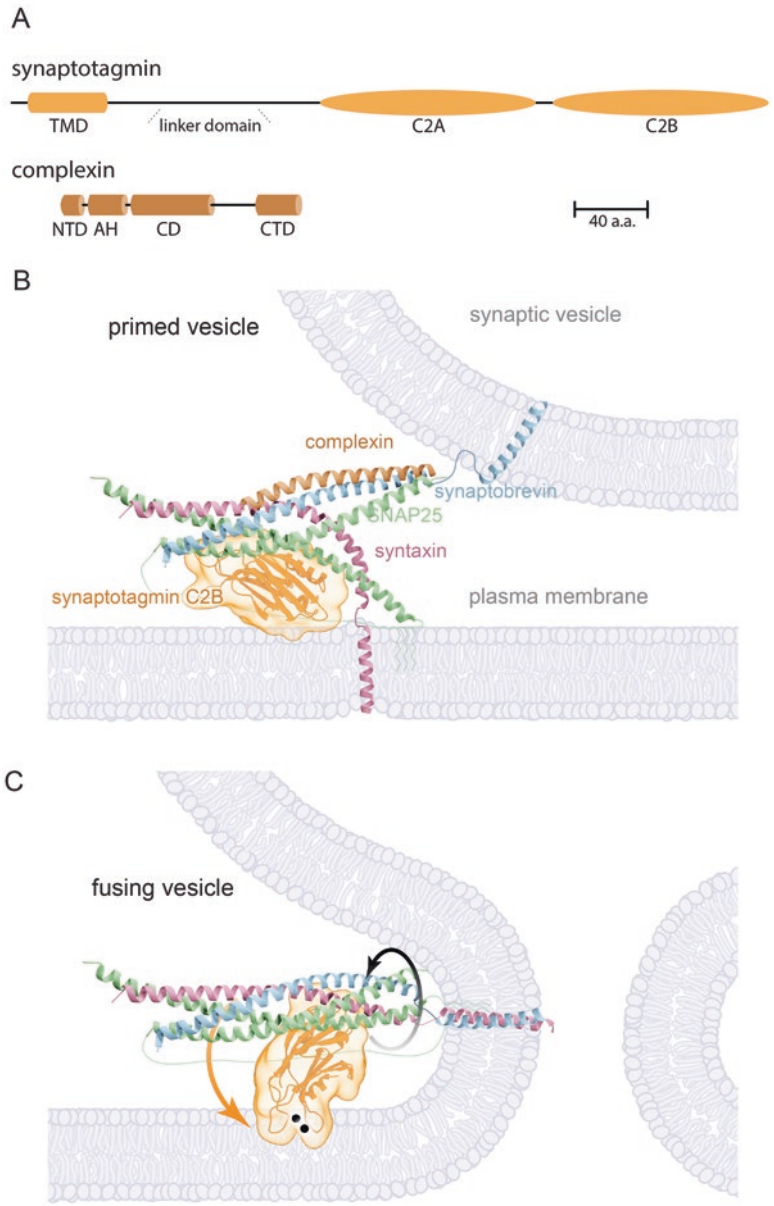

Fig. 5 Calcium disinhibits the paused state. (**a**) *Domain architecture of synaptotagmin and complexin*. Synaptotagmin is attached to synaptic vesicles via a transmembrane domain. A linker domain connects the transmembrane domain to two C2 domains. Complexin binds synaptic vesicles via its C-terminal domain (CTD). The central domain (CD) binds syntaxin and synaptobrevin across the zero layer, stabilizing the primed state. The accessory helix (AH) prevents SNARE zippering. (**b**) *Primed vesicle*. In the partially zippered SNARE complex, the central domain (CD) of complexin (brown) binds the groove between synaptobrevin (blue) and syntaxin (red).

In the horizontal configuration, the C2B domain binds the SNAP25 and syntaxin helices in the SNARE complex [348]. By binding membranes, through the polybasic ventral surface, and SNAREs via the dorsal surface, the horizontally configured C2B domain can serve as a bridge between the plasma membrane and the SNAREs [349]. Low-resolution Cryo-EM structures of C2AB fragments bound to SNARE complexes on lipid nanotubes further support the existence of this configuration [350].

The interaction between C2B and the SNARE complex is likely to mediate both positive and negative functions in membrane fusion. When reconstituted in an in vitro liposome fusion assay, synaptotagmin can act alone as both a fusion clamp in the absence of calcium, and an accelerator of fusion in the presence of calcium [351, 352].

Synaptotagmin likely promotes fusion by docking synaptic vesicles at release sites (Fig. 4a). By binding both the membrane and SNAP-25, synaptotagmin links synaptic vesicles to the plasma membrane [353]. Null mutants in synaptotagmin reduce vesicle docking by half ([354], but see also [95]), and mutations that disrupt either the membrane interface of the C2B domain or the dorsal SNAP25 interface dramatically reduce calcium-triggered evoked release [345, 348, 349, 354–356]. The calcium-independent binding of membranes and SNAREs by the C2B domain likely accounts for the positive role that synaptotagmin plays in fusion.

But this same horizontal configuration of the C2B domain might also inhibit SNARE-mediated fusion by preventing full zippering of the SNARE complex (Fig. 5b). Historically, it was believed that calcium facilitated fusion by triggering synaptotagmin to interact with SNAREs; however, it now appears that calcium instead dissociates synaptotagmin from the SNAREs to allow for rapid fusion [349]. Calcium acts as an electrostatic switch that tips the C2 domain into the membrane [346, 357]. This upright orientation would increase the tilt angle of the SNAREs [358], and might simultaneously break the contacts between the dorsal surface of C2B and SNAP-25 [349], and allow winding to proceed to the C-terminus and fusion of the membranes.

5.4.2 Complexin

Complexin is a small protein (130–150 residues). It consists of four domains: an N-terminal domain, an accessory helix, a central region, and a C-terminal domain that anchors complexin to the synaptic vesicle membrane [359, 360] (Fig. 5a). Complexin is largely unstructured in solution [361] but becomes partially helical upon interacting with membrane and the SNARE complex [362]. Except for a very weak interaction with syntaxin, complexin does not bind individual SNAREs [363]. Rather, the central region of complexin forms an α-helix that binds between syntaxin and synaptobrevin, across the zero layer. The accessory helix of complexin projects between the apposed vesicle and plasma membranes [363, 364]. Full zippering may be indirectly blocked by the steric hindrance between the accessory helix and vesicle membranes [365–367]. Alternatively, the accessory helix could interact with the membrane-proximal C-termini of synaptobrevin and the Qc-SNARE of SNAP25

[368] and stabilize a splayed configuration of SNARE C-termini (Fig. 5b). Irrespective of the precise mechanism, the key role of complexin is to stabilize the half-zippered state (Fig. 5b).

Genetic tests of complexin null mutants in both vertebrates and invertebrates indicate a positive role in vesicle priming. Together with synaptotagmin, complexin holds the vesicle in a primed but paused state. In the mouse complexin double knockout, evoked release is reduced to less than 50% [369–371]. In *Drosophila* knockouts, evoked responses are reduced to 40% [372]. In *C. elegans* knockouts, evoked responses are reduced to 10% [373, 374]. Mutations in the central helix eliminated activity of complexins from each species [374–376]. This positive role is evolutionarily ancient—complexin from sea anemone can rescue evoked responses in the mouse [377]. These data indicate that the positive role for complexin acts by binding the central helix, and stabilizing the SNARE complex.

Interestingly, complexin has a prominent role in stabilizing the docked state in worms and flies. In mammals, this role appears minimal. Complexin mutants in *C. elegans* exhibit a 73% reduction in docked vesicles as determined by electron microscopy [373]. Evoked release can be rescued by expression of constructs that contain the central helix, suggesting that complexin binding to syntaxin and synaptobrevin prevents the SNAREs from unwinding [373, 374]. Likewise, in *Drosophila,* the docked vesicles within the readily releasable pool are reduced by 50% in complexin mutants, and increased to 200% in animals overexpressing complexin [372] (see Box 1 for explanation of the readily releasable pool). In vitro studies show that vertebrate complexin can promote the docked state [378]. However, in mouse complexin mutants, vesicle docking in electron micrographs is not decreased [95, 369], and neither is the readily releasable pool as measured by hypertonic sucrose [369, 375]. In the mouse, stabilization of the docked state is less reliant on complexin, and instead may preferentially use another protein such as synaptotagmin [371, 379, 380]. In *Drosophila,* complexin and synaptotagmin mutants are additive [372]; in mouse, the double mutant resembles a synaptotagmin single mutant [371].

Along with a positive role in priming, complexin also plays a prominent role in inhibiting vesicle fusion in invertebrates. In *Drosophila* and *C. elegans*, knockouts of complexin exhibit *increased* rates of tonic miniature currents [372–374, 381]. Although tonic mini rates in the nematode are calcium-dependent, a fraction of fusions in the nematode complexin mutant are calcium-independent [373]. Inhibition of vesicle fusion is contributed by the N-terminal domain, accessory helix, amphipathic helix and C-terminal anchor [382, 383].

The inhibitory role appears to be minor in the vertebrate central nervous system. In most complexin knockout experiments, spontaneous fusion is unchanged or reduced [361, 362, 377]. However, in complexin knockdowns, an increase in spontaneous vesicle fusions has been observed in cultured neurons [384–387]. These contradictory results may arise due to differential levels of complexin, leading to different levels of vesicle priming. Alternatively, they may result from differences in the balance between the inhibitory and facilitatory functions of complexin in different organisms [362, 365–368].

What is the contribution of complexin to the primed state? When challenged with 500 mM sucrose the size of the readily releasable pool was unchanged in complexin mutants [369, 375]. By challenging with a reduced hyperosmotic challenge, 250 mM sucrose, the readily releasable pool was reduced to 50% in the complexin mutants [388]. Moreover, the profound loss of evoked release in complexin mutants could be restored by increasing calcium [375, 388]. Thus, the docked pool as measured by hypertonic sucrose is normal in total size, but the releasable pool is "reluctant" rather than "ready." These vesicles can only be recruited by either increasing calcium or by potentiating the synapse using multiple stimulations [388]. Similarly, in the calyx of Held, spontaneous release is decreased under resting conditions, but after a burst of action potentials asynchronous release is increased [389]. One possibility is that complexin plays a specific role in superpriming rather than more generally in all priming steps [390]. It is possible that high-frequency stimulation can bypass the requirement for complexin by acting on Unc13 proteins.

5.4.3 Unc13

In addition to its function in recruiting vesicles to fusion sites and opening syntaxin, Unc13 may also act again at the half-zippered stage. Unc13 is required for "superpriming" docked vesicles [391–393], in which the release machinery is poised for rapid fusion [394]. This transition is mediated by disinhibition of Unc13 by diacylglycercol (DAG) binding to the C1 domain or calcium binding to the C2 domain of Unc13 [395]. Superpriming decreases the latency between calcium influx and vesicle fusion and increases vesicle release probability [393, 396]. This transition appears to involve a large physical reorganization of Unc13 and the SNARE complex. Cryo-EM studies suggest that Unc13 can forms rings of six proteins between apposed membranes [256]. Within these rings, Unc13 can adopt two conformations: an upright orientation and a collapsed state. Because Unc13 is attached to both the synaptic vesicle and the plasma membrane, the switch in state should bring the membranes from ~21 nm apart to ~14 nm apart—a situation resembling the Rab GTPase-triggered collapse of the tethering factor EEA1 [397]. How this would alter the configuration of SNAREs and SNARE binding proteins is not known.

5.5 C-Terminal SNARE Zippering and Membrane Fusion

After the calcium-triggered release from the half-zippered state, the final steps are rapid and irreversible, involving only the SNARES and lipids themselves. C-terminal zippering releases the remaining energy stored in the SNARE proteins completing membrane fusion and delivering the membrane-bound cargo. In this section, we will briefly explain the forces preventing spontaneous membrane fusion and then describe how multimerized SNAREs might overcome these forces by guiding lipids

through a conserved set of rearrangements to merge membranes (Fig. 6). Importantly, membrane fusion must take place in an organized fashion so that lipid-bound cargo is not lost through burst membranes. Understanding the rapid interplay between SNAREs and lipids during fusion is experimentally challenging and remains one of the biggest mysteries in vesicle fusion.

Membranes do not spontaneously fuse. Membrane stabilizing forces include the hydrophobic core that minimizes solvent-exposed surfaces, elastic forces that resist

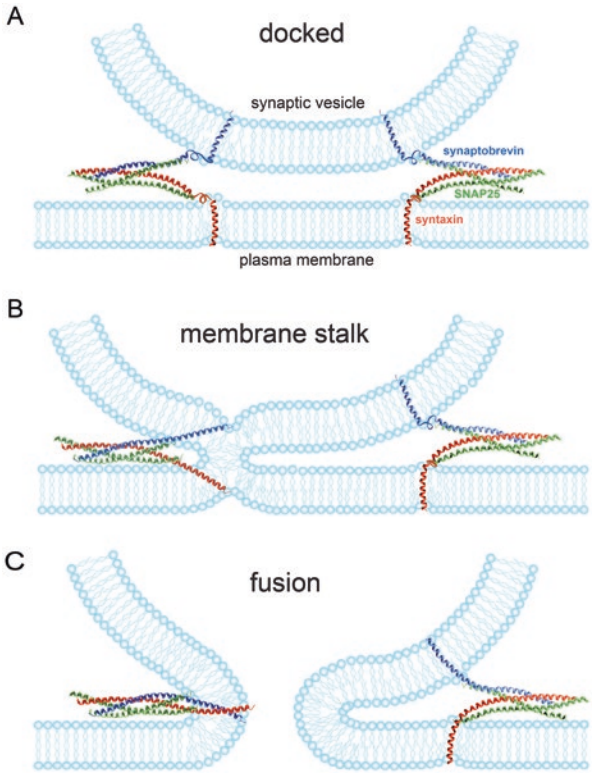

Fig. 6 Steps in membrane fusion. The high repulsive forces between lipid membranes prevent them from fusing. The SNAREs provide the energy that enables the lipid rearrangements required for fusion. This model assumes the SNAREs are not clustered in the center, but rather are in a ring at the edges of a contact zone. Such an arrangement might be required to accommodate large proteins, such as Unc13, associated with the primed state. (**a**) *Docking*. Pairing of the SNAREs brings the membranes into close proximity; winding of the SNAREs is paused at the half-zippered state. The juxtamembrane domains disrupt lipid packing locally but these unstructured regions cannot themselves drive fusion. (**b**) *Stalk formation*. Calcium binding to synaptotagmin (not shown) disinhibits SNARE winding, and propagates helix formation into the flexible juxtamembrane segments. Forcing the helices together brings the locally disrupted lipids to meet and causes the merger of the proximal leaflets into a lipid stalk. (**c**) *Fusion*. Full fusion of the two membranes requires the transfer of energy from the SNARE complex to the transmembrane domains. Coiling of the transmembrane domains of syntaxin and synaptobrevin drive charges at the C-termini into the membrane that cleaves the stalk. For simplicity the figure only shows a single SNARE winding and breaking the membrane. Vesicle fusion requires multiple, most likely flanking, SNAREs to fuse a synaptic vesicle

monolayer deformation, and repulsion generated by negatively charged phospholipid head groups [398, 399]. Charge repulsion, in particular, keeps membranes 1–2 nm apart, and this space must be dehydrated to bring the bilayers together [317]. The lipid rearrangements necessary for membrane fusion and the membrane stabilizing forces that must be overcome were initially predicted from mathematical modeling of pure lipids [400, 401]. Membrane fusion can be broken into three steps: (1) membranes are brought into proximity, (2) membrane deformation allows the merger of the proximal leaflets of the bilayer, and (3) the merger of the distal bilayers completes fusion.

To drive fusion, the stabilizing forces of lipid packing must be disrupted by deforming the membrane. Specifically, point-like protrusions lower the energy of hydration repulsion and enable the formation of a lipid stalk between the proximal leaves of the lipid bilayer [401–404]. Lipid stalks can proceed to full fusion or relax into an extended hemifused state. Intermediates in which the proximal membranes are fused can be observed by the exchange of lipids between membranes without lumenal content mixing [405–408]. In vitro, extended hemifusion intermediates can transition to full fusion [61, 409, 410]. However, in vivo, the lipid stalk likely transitions directly to full fusion [411–413]. These common lipid intermediates are present in all membrane fusion reactions [398, 414–416].

How might the SNAREs fuse membranes? The SNAREs are uniquely suited to overcome the stabilizing forces of membranes: they can force membranes together to dehydrate the intervening space, and they actively disrupt lipids by bending membranes (Fig. 6). Five characteristics of the SNAREs are central to the current models for their function in fusing membranes. First, productive SNAREs assemble in a parallel orientation [45, 50, 53, 167, 417]. Due to their parallel orientation, SNARE assembly leads to the close apposition of the transmembrane domains and hence the membranes themselves (Fig. 6a). Second, the SNARE complex must consist of at least two SNARE molecules with transmembrane domains [418]. The transmembrane domains must be inserted into both of the membranes destined to fuse [55, 56]. Third, SNAREs contain numerous basic residues in their juxtamembrane region that are likely to interact with the negatively charged head groups of lipids. Additionally, the synaptobrevin juxtamembrane region contains tandem tryptophan residues that are likely to insert into bilayers and disrupt their packing. Mutations or alterations in the positioning of these tryptophan residues disrupt fusion both in vitro [419] and in vivo [384, 420–424]. Fourth, the energy released by SNARE zippering is concentrated at the C-terminal end [171], where the transmembrane domains are located. Zippering of the SNARE proteins during core complex assembly transduces force to the transmembrane domains that can overcome barriers to fusion [56, 425]. As SNARE winding propagates to the C-termini, the transmembrane domains will be forced together and bring the lipids of vesicle and plasma membranes together and lead to the formation of a membrane stalk (Fig. 6b). Fifth, SNARE winding propagates into the helical transmembrane domains of synaptobrevin and syntaxin [171, 426–428], and thereby transfers energy generated from SNARE zippering into the vesicle and plasma membranes, and forces them together. Torque

on the transmembrane domains might force dimples in the lipid bilayer at regions of *trans* SNARE complex formation, perhaps corresponding to the point-like protrusions that are thought to be necessary to initiate the fusion of the proximal bilayers [399, 402, 418]. The transmembrane domains are therefore likely to directly disrupt lipids as SNARE assembly proceeds [56, 384, 425]. As the C-termini of the transmembrane domains of synaptobrevin and syntaxin wind around each other, they will merge the distal leaves of the bilayer, and break the barrier between vesicle lumen and extracellular space (Fig. 6c).

Together, these characteristics allow the SNAREs to dehydrate and disrupt lipid bilayers. In in vitro assays, a single SNARE complex is capable of catalyzing fusion [429, 430]. Nevertheless, this result has not been reproduced in vivo and the single SNARE complex is only sufficient to fuse highly fusogenic membranes and, even then, does so with very slow kinetics [429] and without the ability to maintain an open fusion pore [431]. This result is not surprising. Measurements of the energy released from zippering the entire SNARE motif range from 13 k_BT, for yeast exocytic SNAREs, to between 27 k_BT and 68 k_BT, for the neuronal SNAREs [171, 173, 175, 177, 432]. However, C-terminal zippering itself has only been measured to release a maximum of 27 k_BT of energy [173]. These figures are close to the theoretically calculated 40 k_BT to 100 k_BT that is needed for membrane fusion [63], but they are not quite enough. Physiologically, a single SNARE is not enough to fuse membranes. It must be getting help from some friends.

First among those friends are the SNAREs themselves, they form linked rings of complexes. Early electron microscopy studies demonstrated that SNAREs assembled into star-shaped structures with their transmembrane domains at the vertex [433]. Cryo-EM studies by Jim Rothman's group identified six SNAREs complexes sitting beneath a vesicle [434, 435]—a number that precisely matches the optimal number of SNAREs in modeling experiments [436]. Though the interactions are quite weak [437] it has been shown that both syntaxin and synaptobrevin form higher order multimers via conserved regions located in their transmembrane domains [438–440]. At the level of a fusion pore, membranes are largely rigid, thus SNAREs will be mechanically coupled, potentially allowing them to coordinate zippering [434, 436]. By aligning the transmembrane domains of the SNAREs at the vertex, the SNAREs are capable of delineating a patch of membrane where fusion can begin.

In vivo evidence for multimerization comes from a combination of the dose-dependent block provided by peptide blockers and botulinum neurotoxins as well as the cooperative action of the SNAREs themselves [441–447]. Together, these experiments have estimated between 2 and 15 SNARE complexes are needed for productive fusion [441, 444–447]. Titration of syntaxin in neurons indicated a Hill coefficient for cooperativity of 3 for the SNARE complex [90]. These data suggest that the Hill coefficient of 3–4 for calcium cooperativity [448] may not reflect calcium-binding cooperativity within synaptotagmin's C2 domains, but it rather reflects calcium-dependent conformational changes among the SNAREs [443].

5.6 Disassembly of SNARE Complexes

After vesicle fusion, the SNAREs are in the plasma membrane of the active zone in a *cis* SNARE complex. The SNAREs and other components of synaptic vesicles must be cleared from active zone to allow sustained vesicle fusion. The SNAREs must be separated from each other and sorted to their correct compartments. Studies of temperature-sensitive alleles at *Drosophila* synapses indicated that the ATPase NSF acts after membrane fusion during the recovery of synaptic vesicles [36, 449]. NSF does not directly bind the SNARE complex but binds via alpha-SNAP, which either acts as a stator to hold the complex as NSF unravels individual strands, or acts to apply reverse torque to open the complex like uncoiling a stranded rope [187, 199].

How is the *cis* SNARE complex prevented from instantly reforming? The chaperone Unc18 may remain attached to syntaxin during fusion by binding the N-terminal motif. Unc18 binding to syntaxin in the closed state would prevent it from rejoining the SNARE complex [283]. Synaptobrevin is sequestered by AP180 [450]. The EH domain protein intersectin binds synaptobrevin and SNAP-25 [451, 452]. It is not known where in the synapse the SNAREs are separated. NSF may act at the plasma membrane to unwind SNAREs; alternatively, disassembly may take places at synaptic endosomes [453].

6 Summary

Rounds of SNARE assembly and disassembly lie at the center of all vesicular trafficking [454]. Assembly of the SNAREs into a four-helix bundle drives fusion of synaptic vesicles with the plasma membrane and thereby mediates the release of neurotransmitter [455]. αSNAP and the ATPase NSF, meanwhile, survey the landscape for unproductive SNARE assembly—actively disassembling them. The assembly of SNAREs is carefully orchestrated by Unc18 and Unc13 [143]. Unc13 tethers vesicles and opens syntaxin, allowing SNARE assembly to begin. Unc18 provides a template on which syntaxin and synaptobrevin are proofread to ensure correct assembly. Together, Unc13 and Unc18 ensure that productive SNARE complexes are formed. The SNAREs are held in a half-zippered state by complexin and synaptotagmin until calcium triggers full SNARE zippering and membrane merger. After membrane fusion and release of neurotransmitter, the entwined *cis* SNAREs are pulled apart by NSF, which reenergizes the system for further rounds of fusion.

This model is generally accepted; nevertheless, controversies and mysteries still remain. An important lesson from the last few decades is that the strength of protein-protein interactions does not necessarily translate to conserved mechanistic features. For example, the templating role for SM proteins, despite its importance, was notoriously difficult to find due to its relatively transient nature. How complexin and synaptotagmin act on SNARE assembly also remains an enduring enigma, very

likely due to the rapid time scale between calcium entry and fusion. Perhaps the holy grail of mysteries remains how the assembly of SNAREs interacts with lipids to guide them through membrane fusion.

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